

Intra-tumoural blood vessels and hypoxia: targets for treatment and imaging to improve anti-cancer therapies : pre-clinical investigations

Citation for published version (APA):

Landuyt, W. (2002). *Intra-tumoural blood vessels and hypoxia: targets for treatment and imaging to improve anti-cancer therapies : pre-clinical investigations*. [Doctoral Thesis, Maastricht University]. Universiteit Maastricht. <https://doi.org/10.26481/dis.20021219wl>

Document status and date:

Published: 01/01/2002

DOI:

[10.26481/dis.20021219wl](https://doi.org/10.26481/dis.20021219wl)

Document Version:

Publisher's PDF, also known as Version of record

Please check the document version of this publication:

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Chapter V

Summary and general discussion

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V.1. Introductory notes

Expanded vascularization is a prerequisite for continued growth of solid tumours. This important biological event seems mainly regulated through the 'parallel' development of hypoxia, in combination with oncogenic transformation and cell survival selection. Both experimental laboratory and clinical evidence have been supplied for the existence of these tumour characteristics, globally referred to as a highly specific micro-environment. Many published data point at the influence of these characteristics on classical therapy and their association with increased malignancy, metastasis and ultimately prognosis. These include the development of bio-reductive compounds, selective drug-delivery systems, both vessel- and hypoxia-specific gene therapy systems, and anti-angiogenesis and vascular targeting. In parallel, becoming aware of this potential for therapy, researchers aimed to identify the intra-tumoural vessel network and oxygenation status using biopsy- or microprobe-based technologies and non-invasive nuclear medicine or radiology imaging.

The broad background of these important tumour micro-environment aspects is presented in **Chapter I.1**, and sets the scene of the present thesis research.

The investigations were guided by several working hypotheses and questions in relation to the tumour micro-environment (see **Chapter I.2**). Briefly, they related to

- (i) Anti-angiogenesis and vascular targeting are two different approaches, for which the efficacy in general and more specifically the impact of tumour volume was investigated;
- (ii) Since these treatments on their own were not expected to eradicate the whole tumour, whatever its size, vascular targeting was combined with radiotherapy or with anti-angiogenesis;
- (iii) The poor intra-tumoural oxygenation condition was previously exploited (by our research group) to establish an anaerobe bacteria-based therapeutic protein transfer system. Tumour volume-related quality and safety of this selective transfer system, and specifically the further improvement of colonization and protein expression from the combination with vascular targeting, were evaluated;
- (iiii) Finally, based on the potential to modulate the intra-tumoural hypoxic condition, a fast and whole body functional MR imaging methodology was investigated for its feasibility to select tumours for a specific oxygenation treatment.

Assessment of the hypotheses and questions (**Chapter II.1 to Chapter IV.2**), and eventual inter-comparison of the various tumour micro-environment-related research, was done with the rat rhabdomyosarcoma (R1). This tumour originated in the jaw musculature of inbred WAG/Rij rats that received a total body irradiation, and has been established *in vitro* and *in vivo* (subcutaneous implantation) for radiobiological investigations since three decades [1, 2]. Cell kinetic measurements showed a cell cycle duration of about 20 hours, and correspondingly a volume doubling time of 3-5 days [3]. The rhabdomyosarcoma tumour consists of spindle cells, showing atypia and numerous mitoses (see Figure V.1).

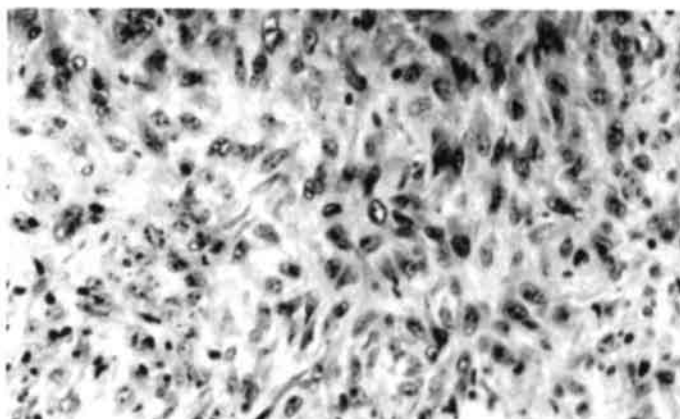


Figure V.1: Representative image (x100) of paraffin-embedded non-treated rhabdomyosarcoma tissue; haematoxylin/eosin staining.

More detailed description using histology and digital subtraction angiography can be found in the description of research results of Chapter II.1 and II.2.

V.2. Tumour blood vessel-based treatment efficacy in relation with tumour volume

The first part of the present pre-clinical studies elaborated the activity of anti-angiogenesis (using the fumagillin analogue TNP-470) and vascular targeting (using the tubulin-interfering combretastatin A-4 phosphate (combreAp)), specifically investigating effects in a broad range of tumour sizes.

Although surely not a general rule for all anti-angiogenic compounds, and although growth delay was definitely longer with small tumours, a transient growth inhibition after repeat subcutaneous injections of TNP-470 was present with tumours having a volume even up to 7 cm³ at treatment start (see **Chapter II.1**). One plausible explanation of this result may be offered by the relation between the relatively rapid tumour growth and accompanying angiogenesis. Most if not all anti-angiogenic compounds show activity against very small tumours (often very much less than 0.5 cm³) only, a growth period during which additional blood vessel formation may be more important for these tumour types. This possibility has however been questioned by Beecken and colleagues, demonstrating about equal effectivity in both poorly vascularized and highly vascularized human bladder carcinoma cell lines growing in severe combined immune deficient (SCID) mice [4]. TNP-470 has been shown to be a strong cytostatic agent with both human and rat endothelial cells [5]. Additionally, TNP-470 showed activity in a broad range of tumour types, both primary lesions and metastasis. The compound was therefore one of the first to enter a clinical phase I trial, and was in several phase II-III trials (e.g. review [6]). Very recently however, the drug has been withdrawn from clinical application. This likely is the consequence of side effects, narrowing the therapeutic window. Severe skin reactions at the site of the repeated subcutaneous injections were observed in our rat study, as was a transient drop in the body weight (10-15 %) at the doses used.

Obviously, drug activity in relation to the tumour type and the site of growth, including quality and quantity of angiogenesis as well as the phase of the angiogenic cascade with which the drug interferes, all need attention when comparing various published data-sets. Yet, based on the volume aspect in our study and taking into account the relatively short doubling time of rodent tumours including xenografts as compared with the temporal evolution of human cancer, the lack of important anti-angiogenic activity in clinical studies is not fully surprising, as very large tumours are involved in human phase I-II studies.

Opposed to the results with TNP-470 anti-angiogenesis is the intra-tumoural activity of the vascular targeting compound combreAp. It is our conviction that a distinction should be made between newly sprouting vessels and the already established yet immature micro-circulation. Indeed, not only is proliferation and migration of endothelial cells necessary to expand vascularization, but of equal importance is the perfect establishment of the endothelial cell cytoskeleton for adequate function within the blood vessel wall. This characteristic (together with the necessity of support cells such as pericytes) could make the difference in

response to agents that specifically target endothelium, as during tumour growth more vessels become established and thus dividing endothelial cells are not exclusively the vulnerable element. Using digital subtraction angiography we were able to visualize the acute and dramatic reduction in the intra-tumoural vascular network evolving in a short time after combreAp treatment. The parallel process of necrosis formation in about the total tumour volume confirmed the idea to kill tumour cells indirectly by cutting off the supply of proliferation nutrients (see **Chapter II.2**). More severe overall intra-tumoural changes from the use of a single intra-peritoneal combreAp injection were anticipated in our studies involving large rat rhabdomyosarcoma tumours as compared to small ones. It does however seem that the effectivity of combreAp differs somewhat among tumour types, with for example the mouse KHT sarcoma [7] and the WAG/Rij rat rhabdomyosarcoma (present thesis research) showing more intra-tumoural damage than the mouse C3H mammary carcinoma [8] or the rat BT4An glioma [9].

Notwithstanding the presence or absence of a difference in morphological outcome, a very important observation with the WAG/Rij rat rhabdomyosarcoma was a clear-cut 'inverse' effectivity of combreAp as compared with the tumour volume-dependent efficacy obtained in general, as well as with the same tumour model, with radiotherapy (see Figure V.2, A *versus* B) or chemotherapy.

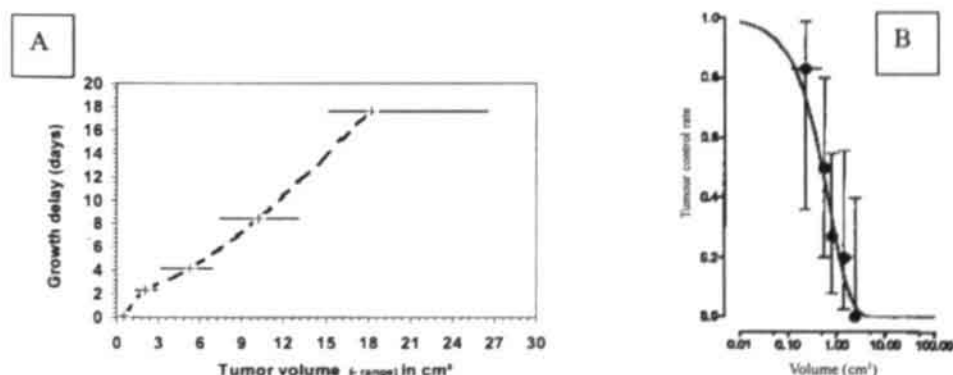


Figure V.2 : A. 'INVERSE' volume-response relationship after combreAp treatment, with respect to e.g. radiotherapy (part B; reproduced with permission).

Indeed with larger tumors ($>6 \text{ cm}^3$) a much stronger effect in terms of growth delay was measured, including some regression shortly after the combreAp treatment (see Chapter II.2, Figure II.2.1), whereas with the very small tumours no significant growth delay was present. Absence of clear-cut growth delays was also observed with the different rodent tumour studies published until now (always involving volumes less than 1 cm^3). A similar finding of tumour volume-related increase in combreAp efficacy has been presented by D.W. Siemann (invited lecture, ESTRO workshop on The Biology of Radiation Oncology, June 2001, Fulgsö, Denmark) using the KHT mouse sarcoma model. In this study, 2-3 logs less cell survival was measured with tumours of 1.5-2 g than with those of only 0.1-0.5 g following a single intraperitoneal injection with combreAp. Our results, and likely those with the KHT sarcoma, strongly suggests a relationship between combreAp activity and the quantity of ill-formed blood vessels established in the larger tumours as compared with the smaller ones. The importance of deficient blood vessel maturation during the establishment of a functional intra-tumoural vascularity has been nicely documented by Benjamin and colleagues [10] and Morikawa and colleagues [11]. These data, demonstrating the control of tumour blood vessel sprouting and maturation through the complex involvement of pericytes, vascular endothelial growth factor and angiopoietins, may help to explain the selectivity of vascular targeting with combreAp and analogues. The information certainly invites further research to exploit the difference between normal host vasculature and the aberrant tumour vessels, a difference that may allow improved anti-cancer therapies.

Of equal potential to explain the 'inverse' tumour size-related effect could be the growing imbalance of viable hypoxic tumour cells in regions with ill-formed vasculature, a fraction that becomes proportionately larger with increase in tumour size, compared with well-oxygenated cells in regions with mature blood vessels. Adding to this explanation is the fact that the combreAp activity not only is directed at endothelium, but seemingly also exerts a cytostatic activity towards tumour cells [12, 13]. This has recently been further investigated and clearly demonstrated by us (Angiogenesis Laboratory, Univ. Hosp. Maastricht) with *in vitro* research involving various human tumour cell lines as well as the R1 rhabdomyosarcoma cells [14]. In fact, using the ^3H -thymidine incorporation assay, the anti-proliferative activity of combreAp was 20-30 times stronger in the human breast (Hs578T) and colon carcinoma (LS174T) cell lines as compared with human umbilical vein endothelial cells. A similar difference of sensitivity was measured in favour of the R1 rhabdomyosarcoma cells as compared with rat heart endothelial cells in these investigations.

Though indications for an anti-angiogenic activity were present in some published research papers on combreAp vascular targeting, we provided direct evidence that such a component plays a role in the overall anti-tumour activity [14]. Indeed, not only inhibition of endothelial cell migration (HUVEC) but also reduced sprouting (BCE) was measured after combreAp application at drug doses that did not interfere with the cell growth (no inhibition of the proliferative activity). Yet, should this activity be most important, the small-sized rhabdomyosarcoma tumours would show a significant growth delay, as was observed with the larger tumours (differential staining of newly formed vessels versus all blood vessels can help to further clarify this item). This deduction is also based on the TNP-470 results, where important growth delays were measured for small rhabdomyosarcoma tumours; yet, we should bear in mind that a different anti-angiogenic pathway is involved (combreAp interfering with the tubulin polymerisation, while TNP-470 inhibits methionyl aminopeptidase-2 activity), and drug-scheduling for both agents was different as well.

Elevated interstitial fluid pressure (IFP) is a known obstacle for the effective drug delivery in solid tumours. The importance to investigate IFP is further highlighted by a recent publication from Milosevic and colleagues, which illustrates that IFP may serve as a significant independent prognostic factor in cervix cancer patients [15]. Part of the research recently published by Eikesdal and colleagues [16], using the subcutaneous implanted BT4An rat glioma, was to assess whether and how the IFP was affected by combreAp and whether such information could add to the understanding of the differential efficacy of the drug in large *versus* small tumours. Our underlying hypothesis was the occurrence of a strong IFP reduction by virtue of the fact that combreAp induces intra-tumoural vascular collapse and vessel number decrease. However, independent of tumour size (range 3-19 cm³), no effect from a single combreAp treatment on the IFP was measured in this rat glioma tumour model. This could eventually reflect the following: the combreAp-induced vascular damage, including vessel permeability changes, could improve diffusion of drug into tumour tissues, which however is counter-balanced by a 'renewed' increase of the IFP from increased vessel leakage. The lack of IFP changes may be explained on the basis of a lesser efficacy of combreAp in the rat glioma (see [9]); however, in the referred study only very small tumours were analysed. At some point of this complex pattern of changes in IFP, it may be of interest to think of self-trapping of combreAp in larger tumours. An extended presence of low doses of combreAp could be more endothelial as well as tumour cell cytostatic than a brief exposure (evidence for the latter can be found through published *in vitro* results). More extensive evaluation in different tumour models including eventually rat rhabdomyosarcoma, is for sure

necessary to enable a conclusion about the impact of IFP changes on the overall tumour response after combreAp treatments. The importance of such studies is accentuated by ongoing studies on combinations with chemotherapeutic drugs.

The discovery of several mechanisms firmly associated with the tumour-selective blood vessel targeting capacity (in the first place), has evolved into small scale clinical research. Currently a limited number of phase I-IB clinical studies in the United States and the United Kingdom examine the impact of combreAp on tumour physiology as well as general compliance and normal organ function. Reports at scientific meetings have provided some encouraging data about the tumour-selective vascular-mediated effects for various tumour types and locations, specifically involving dynamic magnetic resonance imaging of tumour perfusion (Rustin G. *et al*, proceedings of the first International Conference on Translational Research, 2001, Lugano, Switzerland). Very recently, data about these clinical phase I studies have been published, indicating the potent efficacy of repeated cycles of combreAp in the treatment of patients with advanced cancer at doses which did not induce limiting acute side effects [17]. The approaches used to evaluate tumour and normal tissue effects of combreAp treatments indicate vascularity-related anti-tumour activity at doses that do not induce systemic toxicity.

Taking into account the substantial efficacy demonstrated in our (and others) pre-clinical research, as well as the small scale clinical study, and with the necessary caution in mind, the use of such a vascular targeting agent may be beneficial in the first place to de-bulk large tumours. Secondly, vascular targeting may help to improve overall outcome when combined with specifically selected treatment strategies, such as a triple combination using irradiation and either chemotherapy or a hypoxia-based therapy.

The fact that combreAp induces a strong anti-tumour effect at doses below the MTD, generated the search for novel agents with stronger anti-tumour activity. One example is the tubulin-binding agent ZD6126, for which recent data show major selective tumour vascular damage with subsequent hemorrhagic necrosis in both primary and metastatic mouse tumour models, using either a single or repeat injections [18, 19]. However, to which extent the application of this compound results in a broader therapeutic window when compared with combreAp or AC-7700 remains to be firmly documented.

V.3. CombreAp vascular targeting combined with different anti-cancer treatments

Following the single combreAp injection, after which only a peripheral viable rim of cells remained, tumours either continued to grow (small sized ones) or re-grew after a certain time delay (large rhabdomyosarcomas). This was documented with histological analysis for large tumours as peripheral thickenings or nodular outgrowths. With the small rhabdomyosarcomas however, the continuing growth was translated as a broad expansion of the peripheral viable rim. Treatment with a second combreAp injection of these small or large tumours at a selected stage of (re)-growth, resulted in an additional growth delay, however without eradication (see **Chapter II.2**). The failure to fully destroy the tumour, even after a double combreAp treatment, requires this vascular targeting treatment to be combined with other anti-cancer therapies such as radiotherapy.

Since the tumour consists not only of hypoxic tumour cells but at least also of oxygenated proliferating cells, it is obvious to combine radiotherapy with combreAp. The hypothesis is that irradiation given first would kill the oxygenated cells, leaving the hypoxic cells to die as the consequence of vascular targeting induced vessel collapse (see **Chapter III.1.1**). Our data support this elegant idea, as they show an increased growth delay with the rat rhabdomyosarcoma tumour model. Interestingly, these results demonstrate that again the additional anti-tumour effect was more pronounced with large tumours than with the small ones. When combreAp was given before or simultaneous with irradiation, no additional growth delay was observed ; this may be explained on the basis of an increase in the hypoxic micro-environment and thus a larger radioresistant cell population. Others have published similar findings (no enhanced efficacy when combreAp was given before irradiation) with for example a subcutaneous mouse mammary carcinoma [20]. Yet, the proposed theory does not seem to fit the overall response of all tumour types. For example with the mouse KHT sarcoma tumour model also an enhanced efficacy (reduced tumour cell surviving fraction) was seen when combreAp was given before or simultaneous with a single irradiation [7, 20]. These opposing data are difficult to explain unless we assume an important interplay from intermittent acute hypoxia.

When combinations of cytotoxic or cytostatic compounds with ionising radiation are tested for their efficacy, it is usual to consider the presence of a broader therapeutic window as compared with either agent given alone. The published data from Murata and colleagues demonstrate the absence of any increase in radiation-induced acute mouse skin damage

following the combination with combreAp [20]. To further evaluate the important issue of potential increases of radiation-induced normal tissue damage when combreAp is administered in combination with irradiation, we used a mouse gastro-intestinal model. The novel non-invasive methodology that was applied in our experiments specifically assesses the small bowel injury reflected by a reduction of citrulline concentration in plasma, thus offering an estimate of the intensity of induced functional damage (Lutgens L *et al*, proceedings ESTRO meeting, September 2000, Istanbul, Turkey; and [21]). Citrulline is a nitrogen end-product of glutamine metabolism in small bowel mucosa. Combining a single injection of combreAp with total body irradiation at doses from 2 Gy up to 12 Gy did not change the plasma citrulline concentration as compared with the measurements for irradiation only (see Figure V.3).

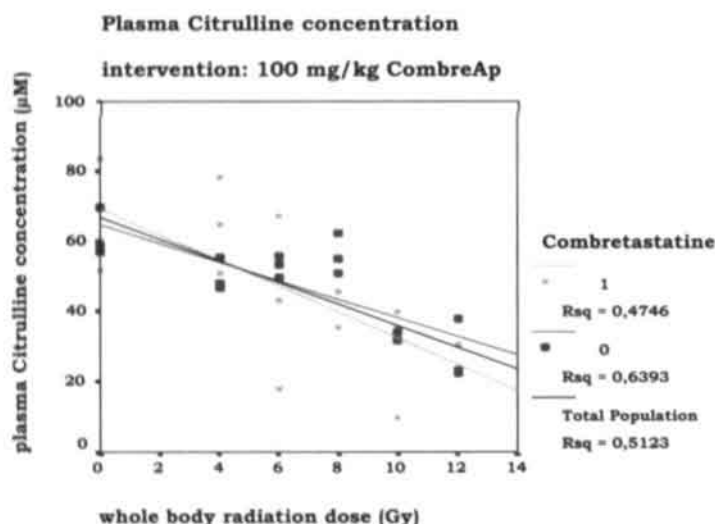


Figure V.3: Data illustrating the lack of a major change in gastro-intestinal damage, measured by the citrulline concentration in plasma, in mice treated with total body irradiation without (0) or with (1) combreAp.

Within the limitations of the selected dosing, sequence and time interval as used in the present investigation, there seems to be a therapeutic gain, specifically when large tumours are considered for such a combination (Landuyt W *et al*, proceedings ESTRO workshop on Biology of Radiation Oncology, June 2001, Fulgsö, Denmark). However, in view of the recently published data on the endothelial cell-involvement with early occurring irradiation

effects in normal mouse intestine [22] and accepting that radiation-damaged normal endothelial cells may become vulnerable for combreAp activity, further research certainly is necessary to determine the most favourable treatment criteria in such combinations, including late effect evaluation. Radiation-induced damage to endothelial cells at clinically relevant doses was demonstrated as early as the 1980's, and the impact of a vascular component in the development of late treatment complications in normal tissues co-determines the therapeutic window.

Our studies combining TNP-470 with combreAp were based on the hypothesis that the further expansion (observed with small tumours) or the re-growth (with larger tumours) after the combreAp treatments should be accompanied by renewed angiogenesis (see **Chapter III.1.2**). The fact that no significant additional growth delay has been observed when TNP-470 was administered during the period of tumour growth could possibly be explained by either an insufficient delivery of the anti-angiogenic drug and/or by a too short duration of TNP-470 application. This contrasts the combination of repeated TNP-470 intraperitoneal injections with hypericin-based photodynamic therapy (PDT), which resulted in a significantly longer growth delay of the murine RIF-1 fibrosarcoma tumour when comparing with the result of either agent alone [23]. Ongoing dedicated studies on differential expression of genes in tumour *versus* normal endothelial cells [24] will certainly aid in defining more efficient combination therapies, for example involving agents interfering with the extracellular matrix modeling process. For sure, also the combination of vascular targeting with agents that interfere with endothelial cell growth simultaneously through several pathways, such as the recently developed anginex [25] deserves further exploration.

Experiments investigating the use of combreAp to further advance the novel anaerobe bacteria-based therapeutic protein transfer to the tumour micro-environment showed a clear-cut beneficial outcome (see **Chapter III.2**). The introduction of the vascular targeting compound was based on the finding of an increase in anoxia and necrosis. This condition should hypothetically favour the *Clostridium* growth and subsequently the expression of the transferred therapeutical protein in the tumour micro-environment. A first part of our research dealt with the evaluation of improving the tumour colonisation with apathogenic anaerobe clostridia (specifically in tumours less than 3 cm³). These small tumours were poorly or not at all colonised with bacteria following spore administration only. CombreAp was injected 4

hours after the systemic administration of *Clostridium* spores, then yielding a bacterial colonisation level almost equal to the one observed with large tumours. The difference in bacterial colonisation between combreAp-treated and non-treated tumours was highly significant, even for tumours much smaller than 1 cm³ ($p < 0.0001$). Of equal importance in those experiments were the results that indicated absence of *Clostridium*-specific antibodies in serum of the treated tumour-bearing rats. The lack of induced immune response allows repeated administration with similar colonization, even if a clinical reason necessitates an intermittent arrest of the bacterial application.

The second series of investigations was carried out to define, separately from the colonisation, the potential improvement of the intra-tumoural expression of the stable therapeutic cDNA construct from the use of a single combreAp treatment. These experiments involved the *E. coli*-cytosine deaminase (CDase). This enzyme converts the non-toxic prodrug 5-Fluorocytosine (5-FC) to the cytotoxic 5-Fluorouracil (5-FU), cloned in-frame with the clostripain promoter into a *E. coli/C. acetobutylicum* shuttle vector. The results clearly indicate stability of the plasmid as well as expression of CDase in all the combreAp-treated tumours. A significantly improved intra-tumoural conversion efficiency of 5-FC to 5-FU was measured (thin layer chromatography), as compared to tumours not treated with combreAp. As expected, based on previous research of our group [26], no proliferating bacteria nor 5-FU were detectable in normal tissues of the combination-treated tumour-bearing rats. Further indication for the *in vivo* anti-tumoural efficacy from this novel combination strategy has been offered by Liu and colleagues [27]. In their study using a mouse squamous cell carcinoma, a stronger growth delay was measured with the *C. sporogenes* CDase-5-FC system as compared with daily systemic 5-FU injection at MTD for 5 days. Combining a vascular targeting compound with the bacterial enzyme-prodrug system may, apart from the advantages presented and discussed in the thesis research (Chapter III.2), improve the anti-tumour efficacy of the 5-FU. Grosios and colleagues [28] indeed demonstrated the enhanced anti-tumour effect in a mouse colon adenocarcinoma from the combination of combreAp with systemic 5-FU treatment.

Our data and the results of research colleagues from the Stanford University School of Medicine (USA) taken together demonstrate the potential of the novel anti-cancer strategy, likely to gain benefit from combination with radiotherapy. Indeed, (i) the chemotherapeutic compound 5-FU is known to 'sensitize' cells for improved ionising radiation efficacy, as demonstrated in many publications (e.g. review [29]). Furthermore, because 5-FU has a high diffusion capacity and because continuous infusion is shown to be the most effective delivery

system, the anaerobe bacteria-based CDase-5-FU treatment strategy with 5-FU production selectively in the tumour micro-environment should permit a beneficial therapeutic window; and (ii) the use of a radiation-inducible promotor of the highly conserved SOS-repair system of *E. coli* (e.g. *recA* gene) further enhances the tumour-selectivity of these anaerobe recombinant bacteria (e.g. [30, 31]). Indeed, this should limit the expression of a therapeutic protein only within the defined, irradiated, tumour volume and in a time-dependent way. This procedure should moreover result in reduced or absent normal tissue reactions which are seen with the classical combination of systemically given 5-FU with radiotherapy.

Other developments in the bacteria-based transfer of therapeutic proteins are offered by the use of e.g. *Bifidobacterium longum* [32] or attenuated *Salmonella* Typhimurium, engineered to express CDase in the tumour micro-environment (e.g. [33]). High tumour to normal tissue ratios were observed in their pre-clinical investigations with mice and large animals, as well as in our rat study [34]. Our study moreover indicates that an optimal 'therapeutic bacteria dose' can be defined and that the tumour-colonizing recombinant *Salmonella* (VNP20047) produced 5-FU selectively in the tumours and not in any normal tissues. These data, together with published results from combining X-ray irradiation (5-15 Gy single dose) with attenuated *Salmonella* (VNP20009), non-recombinant for CDase, thus indicate the potential gain that may be obtained from the combination of recombinant bacteria and irradiation [35]. Even more so in the attenuated *Salmonella* setting, the introduction of a radiation-inducible promoter will advance the spatial control and allow the temporal switch for therapeutic protein expression.

V.4. Non-invasive imaging of intra-tumoural oxygenation

Hypoxia- and blood vessel-related treatments necessitate a firm knowledge of (i) the tumour micro-environment, (ii) the potential modulation of these parameters and (iii) the temporal follow-up of induced changes. The modulation of the intra-tumoural oxygen tension when applying for example either carbogen breathing or recombinant EPO administration, does increase the sensitivity of tumour cells to e.g. radiotherapy, but not equally with all tumours. The end-result of individual tumour screening should therefore enable tailored therapy combinations, and a reduction of the chances of treatment failure, while also avoiding unnecessary addition of treatment.

It is well known that heterogeneity of oxygenation is present in all tumour types. Evidence has been provided using for example the Eppendorf polarographic needle electrodes to measure pO_2 or antibody-based staining of hypoxic cells in pre-clinical and patient studies. These studies also indicated that unfortunately the heterogeneity was also often strongly present within the individual tumours, whatever the type or the site of growth. This heterogeneity has also been observed in the subcutaneous implanted rat rhabdomyosarcoma using immunohistochemical staining and fibre-optic microprobe pO_2 measurements (Oxylite Oxford Optronix, UK). A global and fast picture may allow a more optimal tumour stratification for such individualized treatment. Functional MRI using the BOLD methodology provides a complete non-invasive screening with both spatial and temporal tumour physiology information.

The application of EPI sequences in T_2^* -weighted functional MRI allows us to examine the entire tumour and body in a multi-slice way, and thus provides a comprehensive evaluation of responses to external stimuli. However, the optimal balance between spatial and temporal resolution remains a delicate item when using the T_2^* -weighted imaging in EPI. Spatial resolution is reduced by geometric distortion and susceptibility artefacts related to the air-tissue interface. We demonstrated that a practical and rapid mold technique, using a fast setting alginate, objectively reduced these problems (see **Chapter IV.1**). Indeed, improved T_2^* -weighted image quality and delineation of anatomical structures under investigation was obvious. Separately from the rat tumour study, the objective improvement of image quality was also seen in the larger-animal study of brain function after external stimuli, a result that indicates its broader applicability in this radiological analysis.

Subsequently, the results of a separate series of experiments using BOLD fMRI with GE-EPI (TE39) operating at 1.5 Tesla (see **Chapter IV.2**), and incorporating the mold technique, provided good evidence for the feasibility to image tumour oxygenation changes from carbogen breathing of the host. Changes in intensity of the T_2^* weighted images were very rapidly seen after the start of the carbogen breathing. The data indicate that the quantity of hypoxia modulation was not dependent on the tumour size; inter-, but also intra-tumoural variability was found. An additional advantage of our method is the possibility to detect paradoxical responses to carbogen breathing instantaneously within an individual tumour. The combined analysis of the tumour volume showing intensity changes (voxel number) together with the change in intensity within the responding voxels allowed the selection of those tumours that significantly reacted favourably.

Our functional MR measurements involved a clinical apparatus (1.5 T) and therefore the present application can be considered feasible in terms of patient translation. The BOLD MR methodology has very recently been demonstrated to be a valuable tool to study tumour oxygenation status and modulation in a patient group which involved different tumour types and sites of growth [36]. Though this was a single slice evaluation using T_2^* -weighted gradient-echo imaging on a 1.9 Tesla scanner, the data also indicated inter-tumour variability of carbogen response even for tumours of the same type and similar location. Some tumours showed a high signal intensity change, while for others this was very small. Similar observations were made in pre-clinical rodent tumour models by the same research group as well as other teams using the gradient-echo single slice MR technique.

Not unimportantly, the recent introduction of stronger magnetic fields (well above 2 T) in clinical radiology may broaden the applicability of the fast whole body BOLD fMRI. Indeed, their use should improve resolution (smaller voxel and increased signal-to-noise performance) and thus the modelling of physiological oxygenation changes.

Since the methodology is completely non-invasive, changes can be evaluated in time. This is very important, as some recently published small-scale clinical studies using dynamic MRI indicated the necessity to evaluate tumour micro-environment before and during the course of treatment.

Temporal information in addition to established parameters of tumour response may, whilst gaining prediction of the result of the ongoing treatment, ultimately guide treatment.

V.5. Perspectives

Treatment resistance is, apart from intrinsic cellular characteristics, to an important extent due to the physiologic and biochemical heterogeneity within tumours. Presently, strong interest in morphological and functional parameters that define the difference between tumour and normal tissue vasculature and its relationship with the oxygenation status of tumours evolves into the use of this information for treatment selection and prognosis evaluation.

Throughout the discussion parts of the present thesis research, the potential benefit from using the proposed novel approaches in clinical oncology has been indicated. It is at the same time however realized that several shortcomings still remain to be resolved to optimize such strategies. Also research about the complementary information on tumour oxygenation and its

relation with the vascularity, which can be gained by using different invasive and non-invasive techniques, needs much more in-depth documentation for various tumour types.

The pre-clinical studies demonstrated the disruption of the intra-tumoural blood supply, either through anti-angiogenesis or vascular targeting methodologies, which significantly resulted in tumour cell loss. It was clearly shown in the present thesis research that the combreAp vascular targeting activity was stronger against larger rat rhabdomyosarcoma tumours as compared with small ones. Although this very interesting aspect (the reverse efficacy as observed with for example radiotherapy) has been reported to some extent with the mouse KHT sarcoma model by D.W. Siemann (invited lecture, ESTRO Radiobiology Workshop, June 2001, Fulgsö, Denmark), more information is necessary to explain and to firmly establish this characteristic with other tumour models. Already, the issue of large tumours being attractive for the combreAp vascular targeting activity can be deduced to some extent from the phase I study, data that have been reported very recently [17]. The knowledge about tumour size involvement in vascular targeting treatment will become even more important *when combination therapies are introduced*.

Combining the tubulin-interfering compounds such as combreAp, which are active at doses below MTD, with radio- or chemotherapy or anti-angiogenesis seems promising. But this aspect needs more pre-clinical elaboration before such strategies can safely enter clinical trials. Published results and data offered by the present thesis research indicate an increased anti-tumour effect with single dose combinations. Based on these results, the combination of vascular targeting with ionizing radiation, both fractionated and at clinically relevant doses, can provide a broader therapeutic window but only when the appropriate scheduling is used. The latter has to delicately take into account the different changes in oxygenation resulting from both treatments in order to avoid a reduced efficacy of either agent. The fact that such fractionation combinations can be advantageous towards tumour cell death has been indicated very recently by a mouse KHT sarcoma study of Siemann and Rojiani [18]. Injecting the novel vascular targeting compound ZD6126 twice during a 10-fraction radiation treatment, the authors measured a significantly increased growth delay (with very small tumours at treatment start) as compared with ionising radiation only. Again, such an improvement may be tumour type dependent, as it may relate to differences in oxygenation changes during radiotherapy. Indeed, we did not find a change in the radiation-induced growth delay of the small rat rhabdomyosarcoma when fractionated radiation treatment was combined with the vascular targeting combreAp (abstract 354, proceedings ESTRO meeting, Istanbul, September 2000). The increase in local tumour control from the combined combreAp and fractionated

irradiation had borderline significance in comparison with radiotherapy alone in the C3H mouse mammary carcinoma study of Murata and colleagues (abstract 353, proceedings ESTRO meeting, Istanbul, September 2000).

Furthermore the combination of blood vessel targeting modalities with the classical therapies necessitates the study of normal tissue responses (*cfr. supra*: general discussion). The involvement of blood vessel damage induced by ionizing radiation has been well recognized to play a major role in the development of consequential as well as late occurring normal tissue defects. The recently published data on intestinal crypt damage in mice demonstrate the involvement of radiation-induced endothelial lesions also in the evolution of this acute dose-limiting side effect [22]. Absence of an increased radiation-induced acute (*e.g.* gut) and late (*e.g.* lung) normal tissue effect has been suggested by us (*cfr. supra* Chapter V) and by Horsman and colleagues (presentation at ESTRO meeting, Prague, September 2002). Yet, these studies involved single dose treatments only, as well as a single time interval between both agents. Therefore, results from experiments including fractionation and prolonged drug use, including different sequencing, are of major importance for safety and eventual establishment of the therapeutic window. Of interest in this context are the results published by Park and colleagues, describing the increased mitogen activated protein kinase-dependent VEGF expression in glioblastoma cells but also in primary astrocytes and this following exposure to a radiation dose of 2 Gy [37]. The understanding of gene expression changes that occur after irradiation, such as VEGF, will further improve the combined use of radiotherapy with either vascular targeting or anti-angiogenesis or even the triple combination.

An additional hypothesis that deserves stringent research is the combination of vascular targeting with bioreductive compounds. The latter may indeed kill tumour cells in transient hypoxic condition that is induced by the vascular targeting activity. Although normal tissue toxicity could limit the applicability, as reported by Lash and colleagues [38] when however DMXAA was combined with various bioreductive drugs, the type of combination remains attractive and should be examined with the tubulin-interfering systemically less toxic compounds such as combreAp. Such a treatment may circumvent the reduced efficacy of classical therapies that is the result of hypoxia-related resistance and changes to a more malignant phenotype (the latter problems are nicely reviewed by *e.g.* [39]).

As indicated in the general discussion, several directions to improve the bacteria-based therapeutic gene transfer need experimental evidence. One of these involves the non-invasive assessment of the intra-tumoural expression of the transferred active protein. Regarding the CDase-5-FC strategy, the non-invasive ^{18}F MRS is the most attractive methodology. The

potential utility has already been demonstrated in other treatment conditions both in vitro and in vivo; for instance to measure the 5-FU metabolism following the systemic injection of the chemotherapeutic drug, or to evaluate the conversion of 5-FC to 5-FU by the CDase (*e.g.* [40-42]). The parallel evaluation of the 5-FC conversion and the growth changes in tumour bearing rodents is under investigation by us.

Due to the ongoing development of tumour vessel-based therapies and due to the need for non-invasive screening of individual tumours before, during and after any therapy, the refinement of functional and contrast-based dynamic MRI and PET is a pre-requisite in oncology. Specifically the evaluation of the complementary information that may be gained on tumour micro-environmental characteristics and their modulation when using different methods of analysis will enable treatment guidance and reduce the chances of failure. This could be possible by relating for example the oxygenation and metabolic condition with the vascular function and with the proliferation characteristics for an individual tumour and for different tumour types. These aspects have been indicated to some extent in the literature, as well regarding the tumour micro-environment (*e.g.* [43-45]) as in relationship with the MRI evaluation of vascular targeting effects [46, 47]. The bottom line: a close-to-ideal concept for the future planning is to further evaluate the efficacy of novel anti-tumour strategies using stringently documented and complementary non-invasive imaging methodologies.

V.6. References

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Chapter VI

Samenvatting

Curriculum Vitae

Lijst van publicaties (vanaf 1998)

Samenvatting

De intense celdelingsactiviteit inherent aan de groei van tumoren noodzaakt een afdoende en constante beschikbaarheid van voedingsstoffen en zuurstof. Initieel zorgen gecoopteerde en nabijgelegen vaatstructuren voor deze aanvoer, maar al snel ontstaat een on-evenwicht met de tumoruitbreiding wat resulteert in hypoxische regio's. Via de productie van hypoxie-gemedieerde angiogene factoren wordt de ontwikkeling van nieuwe bloedvaten uit de bestaande vasculatuur geïnduceerd. Op deze wijze kunnen tumorcellen overleven en kan het proces van celproliferatie voortgaan of hervatten. Juist deze verschillende belangrijke stappen in de vicieuze cirkel van lokale tumorexpanisie zijn het aanknopingspunt voor nieuwe methoden in behandeling en diagnose van vaste tumoren.

Ook het onderzoekswerk beschreven in de voorliggende thesis relateerd gebruikt karakteristieken van de tumorale micro-omgeving voor het testen van gerichte therapie en diagnose.

In **hoofdstuk I.1** worden specifieke parameters van de tumorale micro-omgeving gedefinieerd.

In de eerste plaats en voornamelijk in relatie met het gepresenteerde onderzoek, zijn een aantal aspecten van tumorale vaat-gerichte behandelingen in het kort geschetst. Steunend op de tot hiertoe gekende actiemechanismen wordt onderscheid gemaakt tussen 'anti-angiogenese' en 'vascular targeting'. Produkten met anti-angiogene eigenschappen verhinderen de vorming van nieuwe bloedvaten, terwijl vascular targeting stoffen selectief de bestaande, slecht ontwikkelde, intra-tumorale bloedvaten beschadigen en vernietigen.

Naast de enkelvoudige tumorbehandeling met zulk een produkt, wordt de potentiële verruiming van het therapeutisch venster *via* een combinatie met een klassieke behandelingsmethode (bijvoorbeeld radiotherapie) in het kort besproken.

Verder wordt het belang en het evalueren van hypoxie in vaste tumoren uitgelegd in functie van tumor evolutie en behandeling. Vooral wordt aandacht besteed aan invasieve versus niet-invasieve methoden om hypoxie in kaart te brengen.

Aansluitend worden de belangrijkste behandelingsmogelijkheden gericht op de intra-tumorale hypoxie, zoals het toedienen van bioreductieve produkten, bondig voorgesteld. Specifiek wordt de achtergrond voor het gebruik van anaerobe apathogene clostridia als transfersysteem van een therapeutisch proteïne naar de tumorale micro-omgeving uitgelegd. Tevens wordt de

mogelijkheid van hypoxie modulatie door gebruik van bijvoorbeeld een zuurstofrijk gas of een vasodilatator belicht.

Tot slot worden in **hoofdstuk 1.2** de doelstellingen met inhoudelijke hypothesen voor het thesisonderzoek geschetst.

Hoofdstuk II beschrijft de resultaten van *in vivo* experimenten enerzijds met de angiogenese-inhibitor TNP-470 en anderzijds met de vascular targeting stof combretastatin A-4 fosfaat. Ondermeer omwille van het verschil in specifieke actie voor beide bloedvat-gerichte behandelingen is het zeer belangrijk hun effecten in tumoren van verschillende grootte te onderzoeken. Voor dit onderzoek werd de syngene WAG/Rij rat rhabdomyosarcoma tumor (R1 cellijn) gebruikt, waarbij longitudinaal voor de verschillende experimenten een onderhuidse inplanting van 1 mm³ stukje weefsel in de abdominale flank gebeurde. Tumor volumina variërend tussen 0.1 cm³ en meer dan 20 cm³ werden beoogd, en als volgt ingedeeld: <1 cm³ (zeer klein), 1-3 cm³ (klein), 3-7 (medium), 7-14 cm³ (groot) en >14 cm³ (zeer groot).

Een significante groeivertraging ($p < 0.01$) werd gemeten na het onderhuids toedienen (om de twee dagen, 5x) van de maximum tolereerbare dosis TNP-470 bij tumoren kleiner dan 7 cm³ (**hoofdstuk II.1**). Er was geen verschil in groeivertraging voor de kleine tumoren tegenover de medium groep. Bij de tumoren groter dan 7 cm³ werd een groeivertraging slechts vastgesteld wanneer de TNP-470 dosering gepaard ging met een ernstige lokale huidlaesie op de plaats van de onderhuidse inspuiting alsmede systemische toxiciteit onder de vorm van een blijvend gewichtsverlies van ruim 15 %. Een lagere dosering minder frequent gegeven (minimale en transiente toxiciteit), induceerde groeivertraging enkel bij rhabdomyosarcoma tumoren kleiner dan 7 cm³. Rechtstreeks vergelijk met gepubliceerde resultaten en dus met andere *in vivo* tumor modellen is niet mogelijk, aangezien deze pre-klinische studies enkel bij tumoren kleiner dan 1 cm³ werden uitgevoerd. TNP-470 bleek evenwel in deze studies zeer actief te zijn bij zowel primaire tumoren (syngene en humane xenografts) als bij metastasen. Ook klinische studies (fase I tot III) toonden belangrijke anti-tumorale effecten door toedienen van TNP-470. Recent werd het klinisch gebruik van deze angiogenese remmer echter stopgezet, waarschijnlijk omwille van een ongunstige balans tussen intra-tumorale activiteit en ernstige nevenwerkingen (*cfr. supra* onze studie).

In **hoofdstuk II.2** worden de resultaten beschreven van de *in vivo* experimenten met het onderhuids groeiend rat rhabdomyosaroom na behandeling met de vascular targeting stof combretastatin A-4 fosfaat (combreAp), een intracellulair tubuline-interfererend molecule.

Het toepassen van vascular targeting als anti-kanker behandeling is op zich een gekende strategie. Vooral hyperthermie, en ook fotodynamische therapie, zijn niet enkel rechtstreeks tumor celdodend maar zijn tevens gekenmerkt door het aanbrengen van bloedvatschade. Belangrijke beperkingen van deze modaliteiten zijn (i) dat alleen oppervlakkige tumoren (of intra-operatief bereikbare tumoren) kunnen worden behandeld, en (ii) het afwezig zijn van selectieve activiteit in het tumorweefsel. Het gebruik van producten die systemisch worden toegediend en selectief de tumorvaten beschadigen, zoals combreAp, kunnen een bredere toepassing kennen.

Na een éénmalige intraperitoneale inspuiting van combreAp aan 1/3 MTD is een snelle en sterke vermindering van het aantal bloedvaten zowel histologisch als angiografisch gedocumenteerd. Deze ernstig vaatschade werd gevolgd door een uitgebreide necrosevorming, ongeacht het tumorvolume op het ogenblik van de combreAp inspuiting. De door combreAp aangebrachte intra-tumorale vaatschade en necrose kan best worden vergeleken met de resultaten na gebruik van tumor necrosis factor alfa (TNF α). Een belangrijk verschil evenwel is de afwezigheid van systemische toxiciteit bij een combreAp dosering met toch een sterk anti-tumoraal effect.

Deze stevige combreAp-geïnduceerde schade werd echter niet vertaald in een algemene groeivertraging of regressie van de behandelde tumoren. Bij zeer kleine en kleine tumoren (<3 cm³) werd geen significante verandering in de groeisnelheid gemeten; de grotere tumoren (vooral deze >7 cm³) vertoonden een zeer significante groeivertraging (p=0.001). Deze tumor volume-afhankelijke resultaten zijn precies het omgekeerde van de resultaten na radio- of chemotherapie. Bij deze laatste behandelingsmodaliteiten zijn grotere tumoren veel slechter of niet behandelbaar vergeleken met de kleine, en dit vooral door de ontwikkeling van inefficiënte vasculatuur en daarmee gepaard gaande hypoxie. Het feit dat grotere tumoren vatbaarder zijn voor combreAp op basis van een belangrijker aantal slecht gevormde bloedvaten kan een uitleg zijn voor deze omgekeerde resultaten in vergelijking met radio- en chemotherapie. Mogelijks zijn in grotere tumoren een grotere proportie tumorcellen afhankelijk van elk intra-tumoraal bloedvat, of zijn veranderingen in interstitiële druk mede de oorzaak van deze resultaten. Andere veronderstellingen die de volume-respons relatie mede kunnen verklaren zijn gerelateerd aan een hypoxie-gemedieerde verandering van de

combreAp activiteit of aan een direct cytostatisch effect van de stof voor de tumorale cellen. Vergelijkingen met literatuurgegevens betreffende tumor groeivertraging of tumorcontrole na combreAp behandeling zijn niet evident, want in zowat alle bekende studies werden alleen zeer kleine tumoren onderzocht.

Het gebruik van een dubbele combreAp toediening (met 1 week interval) resulteerde, zij het minder duidelijk dan bij de éénmalige toediening, eveneens in een tumorvolume-afhankelijke effect. Grotere tumoren waren ook hier vatbaarder voor combreAp dan kleinere. Een bijkomende bevinding was dat kleine tumoren dus ook een groeivertraging vertoonden na een herhalingsbehandeling met een gelijke dosis combreAp.

Hoofdstuk III toont de resultaten van verschillende reeksen experimenten waarbij combreAp werd gecombineerd met een andere vorm van anti-kanker behandeling.

Zoals duidelijk is uit de gegevens beschreven in hoofdstuk II.2, induceert combreAp als monotherapie niet een algemene necrose en is er dus geen tumor eradicaatie. Aan de rand van de rhabdomyosarcoma tumoren bleven een aantal tumorcellen overleven, en is hergroei vanuit die regio's dan ook logisch.

De combinatie met ioniserende straling is uiteraard de eerste keuze. Dit houdt verband met de hypothese dat cellen in de buitenste schil van de tumor voldoende van zuurstof en voedingsmiddelen zijn voorzien, en dus stralingsgevoelig zijn. De resultaten wijzen op een duidelijk tumor volume-afhankelijk effect (**hoofdstuk III.1**): bij grote tumoren ($>7 \text{ cm}^3$) werd een belangrijke bijkomende groeivertraging gemeten in vergelijking met de vrij effectieve bestraling alleen. Kleinere tumoren ($<3 \text{ cm}^3$) vertoonden een niet-significante of afwezige verandering van de groeisnelheid. In al deze experimenten werd de éénmalige bestraling eerst gegeven, om intra-tumorale inductie van hypoxie door combreAp (en dus stralingsresistentie) te vermijden. Het gebruik van combreAp vooraf aan de bestraling, of zelfs gelijktijdig gegeven, leidde niet tot een verlengde groeivertraging.

Tijdens de hergroei van de rhabdomyosarcoma tumoren was er een duidelijke toename van het aantal bloedvaten (vooral perifeer), zoals in beeld gebracht met de digitale subtractie angiografie techniek (zie hoofdstuk II.2). Het verhinderen van deze revascularizatie zou moeten leiden tot een afremming van de tumorgroei. Op basis van deze hypothese werd 1 dag na combreAp de angiogenese remmer TNP-470 gedurende 1 week driemaal toegediend. Deze

reeks experimenten (**hoofdstuk III.1**) wees op een zeer beperkte bijkomende groeivertraging, ongeacht het tumor volume bij de start van de behandelingen. Mogelijks is de afwezigheid van een duidelijk effect het gevolg van een nog té geringe hergroei van de vasculatuur bij de meeste tumoren gedurende de eerste week na de combreAp toediening.

De combinatie van combreAp met een nieuwe recent ontwikkelde anti-kanker strategie, met name het gebruik van anaerobe apathogene clostridia met selectieve transfer van therapeutische proteïnen naar vaste tumoren, werd eveneens onderzocht (**hoofdstuk III.2.A en B**). Onder andere binnen onze onderzoeksgroep werd dit bacterieel systeem met *Clostridium* op punt gesteld voor transfer van het suicide gen cytosine deaminase (CDase) en het cytokine tumor necrosis factor (TNF) α . Met deze combinatie werd een verbreding van het bacterieel systeem-gerelateerd therapeutisch venster beoogd op basis van intra-tumorale kolonisatie verbetering. De hypothese was dat combreAp necrose induceert, ook in zeer kleine tumoren, waardoor de kolonisatie en potentieel ook de proteïne expressie wordt geoptimaliseerd. Daarnaast werd binnen het kader van therapeutische optimalisatie ook gekeken naar mogelijke *in vivo* toxische effecten. Het gebruik van combreAp resulteerde in een uitstekende *Clostridium* kolonisatie van alle tumoren, ook de zeer kleine, equivalent aan de kolonisatie gemeten in grote tumoren niet behandeld met combreAp. In een parallelle reeks experimenten werd de afwezigheid van een duidelijke immuunreactie aangetoond: niet-significante *Clostridium*-specifieke antilichaam concentratie in serum van de proefdieren en geen stijging van de rectale temperatuur. Tevens werd aangetoond dat, indien nodig, de clostridia kunnen worden verwijderd met een specifiek antibioticum zoals metronidazole. De verdere evaluatie van een mogelijke winst door het combineren van combreAp met het anaerobe bacterie transfer systeem gebeurde door het meten van de intratumorale expressie van het CDase. Dit enzyme zorgt voor de omzetting van het schimmelwerend 5-Fluorocytosine (5-FC) tot het cytotoxisch 5-Fluorouracyl (5-FU). De combinatie van combreAp met de clostridia recombinant voor het CDase resulteerde in een stabiele en sterk verbeterde proteïne expressie in alle onderzochte tumoren. De CDase expressie was, zoals geanticipeerd werd, afwezig in al de onderzochte normale weefsels van de met deze combinatie therapie behandelde tumordragende proefdieren.

Het is nuttig de resultaten bekomen met de bloedvat gerichte behandelingen, al dan niet in combinatie met een klassieke anti-kanker therapie, in iedere tumor longitudinaal te kunnen volgen. Het is eveneens noodzakelijk dat een selectie van tumoren kan gebeuren voor

een optimaal gebruik van bijvoorbeeld het anaerobe bacterie-gemedieerde therapeutisch proteïne transfer systeem. Een snelle en degelijke selectie van tumoren die potentieel in aanmerking kunnen komen voor hypoxie-modulerende stoffen zoals hyperoxische gasen en vasoactieve produkten is eveneens gewenst. In dit kader is het gebruik van een niet-invasieve screening techniek zoals magnetische resonantie beeldvorming (MRI), welke zowel snel als volledig tumor-omvattend is, zeer belangrijk. In **hoofdstuk IV** wordt een specifieke toepassing van MRI, gesteund op "blood oxygen level dependent" (BOLD) endogeen contrast, voor het evalueren van tumor hypoxie en de modulatie hiervan met carbogeengas (95 % O₂ + 5 % CO₂) onderzocht. Dit onderzoek gebeurde met een in de klinische radiologie gebruikt apparaat, een totaal lichaam 1,5 Tesla MRscanner.

Het toepassen van snelle sequenties (hoge temporale resolutie), zoals mogelijk met echo planar imaging (EPI), resulteert echter in een verlies van spatiale resolutie. De bekomen suboptimale kwaliteit van de T₂*gewogen beelden is ook het resultaat van geometrische distortie ten gevolge van susceptibiliteits-artefacten, zoals die kunnen ontstaan op lucht-weefsel overgangen. Door gebruik te maken van een soepel blijvend alginaat, dat over het te evalueren lichaamsdeel werd aangebracht, kon een objectieve verbetering van de beeldkwaliteit worden bekomen (**hoofdstuk IV.1**). Deze gegevens tonen duidelijk de winst voor functionele MRI (fMRI) door gebruik te maken van zulk een alginaat in zowel oncologisch als neurologisch onderzoek bij proefdieren.

In een aparte reeks experimenten werd de haalbaarheid en gevoeligheid van de fMRI techniek getest bij WAG/Rij ratten met het onderhuids rhabdomyosaroom (**hoofdstuk IV.2**). Met deze methode werden meerdere sneden gemaakt doorheen het volledige volume van de individuele tumoren, terwijl de proefdieren of met lucht of met carbogeen werden beademd. De evaluatie van de voxelintensiteit, als vertaling van de BOLD veranderingen ten gevolge van carbogeen beademing, was voor alle tumoren mogelijk doorheen het ganse volume. De lage signaal-ruis ratio werd gecompenseerd door het snelle opeenvolgend herhalen van de metingen en door analyse van deze metingen met een aangepast statistisch pakket (SPM96). De T₂*gewogen beelden toonden verschillen in intensiteitsveranderingen ten gevolge van carbogeen, zowel binnen dezelfde tumor als tussen de verschillende tumoren (ook deze met een vergelijkbaar volume). De verandering in intensiteit was zeer acuut na de start van de carbogeen beademing, maar echter niet voor alle tumoren even sterk. Tevens had het gebruik van carbogeen bij enkele tumoren een negatief effect (vermindering in signaalintensiteit), wat

kan worden toegeschreven aan een 'stelen van zuurstof' binnen de tumor of binnen het omliggende weefsel. Op basis van de gecombineerde metingen van (i) het volume, aantal voxels, van intensiteitsveranderingen en (ii) de grootte van deze intensiteitsverandering kunnen individuele tumoren met een globaal positief antwoord op carbogeen beademing worden uitgezocht. De niet-invasieve fMRI methode blijkt een snelle evaluering van de modulatie van hypoxie toe te laten, en dit voor het totale tumorvolume.